Practical procedure for enzyme immunoassay of progesterone in bovine serum

Toshihiko Nakao

Department of Obstetrics and Gynaecology, School of Veterinary Medicine, College of Dairying, Rakuno Gakuen University, Ebetsu-shi, Hokkaido 069-01, Japan

Abstract. An enzyme immunoassay of progesterone was established by using β-galactosidase from E. coli as a label. The enzyme was conjugated with 11α-hydroxyprogesterone-hemisuccinate using water-soluble carbodiimide. Rabbit antiserum to 11α-hydroxyprogesterone-hemisuccinate-bovine serum albumin was previously obtained and anti-rabbit gamma globulin goat serum was used as second antibody. The enzyme activity was measured by utilizing hydrolysis of O-nitrophenyl-β-D-galactopyranoside. The least detectable concentration of progesterone was 12 pg per tube. The measurable range of progesterone in 0.1 ml of bovine serum was between 0.25 ng/ml and 10 ng/ml.

This method satisfied the general criteria regarding specificity, precision and recovery rate. Correlation between the progesterone levels determined by enzyme immunoassay and radioimmunoassay was quite high (r = 0.99, P ≤ 0.01). The present enzyme immunoassay can be applied for practical and routine analysis of serum progesterone.

Progesterone secreted mainly from the corpus luteum plays an important role in human and mammalian reproduction, especially in preparing for nidation for maintaining pregnancy, and in bringing about normal external oestrous activity in conjunction with oestrogen.

Therefore, progesterone levels in the peripheral blood provides very useful information for the diagnosis of pregnancy in some species (Robertson & Sarda 1971) and for the indication of causes of the female infertility and/or subfertility.

Radioimmunoassay has been the most reliable and practical method of determination of progesterone. The radioimmunoassay, however, has some disadvantages, mainly due to restrictions on the use of radioisotope. A great number of small laboratories have not been equipped with the facilities required for radioimmunoassay. This is the main reason why progesterone analysis has not been performed as a routine work in many small laboratories.

Immunoadnaosys using enzyme as a label in replace of radioisotopes have been introduced recently for estimation of steroids (Van Weemen & Schuurs 1972; Dray et al. 1975; Comoglio & Celada 1976; Tateishi et al. 1976; Joyce et al. 1977; Numazawa et al. 1977; Ogihara et al. 1977) and polypeptide hormones (Van Weemen & Schuurs 1971; Miyai et al. 1976).

Dray et al. (1975) were the first to report enzyme immunoassay of progesterone in which β-galactosidase from E. coli was used as a marker of antigen.

The following trivial names are used:
Androstenedione: androst-4-ene-3, 17-dione.
11-deoxycorticosterone:
  21-hydroxy-4-pregnen-3, 20-dione.
11α-hydroxyprogesterone:
  11α-hydroxy-4-pregnen-3, 20-dione.
17α-hydroxyprogesterone:
  17α-hydroxy-4-pregnen-3, 20-dione.
20α-hydroxyprogesterone:
  20α-hydroxy-4-pregnen-3, 20-dione.
5α-pregnandione: 5α-pregnane-3, 20-dione.
Pregnenolone: 3β-hydroxy-5-pregnen-20-one.
Progesterone: 4-pregnen-3, 20-dione.
Although the sensitivity of the assay was as high as radioimmunoassay the evaluation of the practical value of the method in measurement of blood progesterone was not fully elucidated. Later, a more detailed technique of enzyme immunoassay of progesterone was described by Joyce et al. (1977) who used horseradish peroxidase as a label of antibody. The sensitivity of the assay was significantly lower than the method of the previous investigators.

In this paper, a method of progesterone enzyme immunoassay using β-galactosidase, which is specific and highly sensitive and also is applicable for practical routine assay of progesterone in serum is described.

Materials and Methods

1. Reagents and samples

Progesterone was purchased from Merck. Other steroids were from Sigma. 11α-hydroxyprogesterone-hemisuccinate was kindly supplied by Dr. A. Kanbegawa of Teikoku Hormone Mfg. Co. Ltd., Tokyo. β-Galactosidase from E. coli (EC 3.2.1.23) suspended in 2.2 M ammonium sulphate (5 mg/ml) was obtained from Boheringer Mannheim Yamanouchi, Tokyo. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-hydrochloride was purchased from Calbiochem. 2-Nitrophenyl-β-D-galactopranoside was from Merck and 2-mercaptoethanol from Fluka. Petroleum ether (analytical grade) was from Kishida Chemical Co. Ltd., Tokyo.

Blood samples were collected via the jugular vein of two dairy cows in the follicular phase and in the luteal phase. Blood was also taken from 17 cows with cystic ovarian degeneration. The samples were left at room temperature (20°C–25°C) for 3 h. They were then centrifuged at 3000 r.p.m. for 10 min to collect serum. Serum samples thus obtained were stored at −20°C until analysis.

2. Buffer

0.1 M phosphate buffer pH 7.0 used for the assay contained 70 mM disodium hydrogen phosphate, 30 mM sodium dihydrogen phosphate, 1 mM magnesium sulphate, 0.2 mM manganese sulphate and 2 mM magnesium disodium ethylenediamine tetraacetate. The buffer for antiserum dilution further contained 0.06% of bovine serum albumin, 0.05% of bovine gamma globulin fraction II and 0.1% Na3.

3. Anti-progesterone rabbit serum

Antiserum to 11α-hydroxyprogesterone-hemisuccinate-bovine serum albumin raised in rabbit was donated by Dr. A. Kanbegawa of Teikoku Hormone Mfg. Co. Ltd., Tokyo.

4. Anti-rabbit gamma globulin goat serum (second antibody)

Gamma globulin fraction of rabbit serum was collected by precipitation with saturated ammonium sulphate, centrifugation at 7000 r.p.m. for 10 min and dialysis against 0.02 M phosphate buffer pH 7.2 for 12 h. A castrated male goat weighing about 30 kg was first injected sc with 13 mg of the rabbit gamma globulin mixed with 10 ml of Freund’s complete adjuvant. Two months later, 6 mg of the rabbit gamma globulin mixed with 5 ml of the adjuvant was injected sc (booster injection), and this was repeated once a month. Meanwhile, 50 ml of blood was taken via the jugular vein once a week, starting on the 7th day from the first booster injection. The antiserum obtained after the second booster injection was used for the assay.

5. Determination of β-galactosidase activity

Activity of β-galactosidase was measured by a modification of the method of Dray et al. (1975).

The enzyme suspended in 2 ml of 0.1 M phosphate buffer pH 7.0 was incubated at 45°C for 3 h with 20 mM O-nitrophenyl-β-D-galactopyranoside containing 0.7% of 2-mercapto-ethanol. The reaction was stopped by addition of 1 ml of 1 M sodium carbonate. The absorbance was measured at 420 nm on a spectrophotometer (Hitachi-101). Enzyme units were not calculated, and β-galactosidase activity was represented by the absorbance. This simplifies procedures for a preparation of standard curve and calculation of progesterone concentration in serum.

6. Preparation of 11α-hydroxyprogesterone-hemisuccinate-β-galactosidase conjugate

11α-hydroxyprogesterone-hemisuccinate was conjugated with β-galactosidase using water-soluble carbodiimide as described by Dray et al. (1975).

Five mg of 11α-hydroxyprogesterone-hemisuccinate was first dissolved in 2 ml of methanol. After adding 8 ml of distilled water, methanol was evaporated. Water was further added to make a final volume of 10 ml. The pH was adjusted to 4.7. 5 mg of β-galactosidase suspended in 1 ml of 2.2 M ammonium sulphate was centrifuged at 4000 r.p.m. for 15 min. A supernatant was discarded, and the precipitate was dissolved in 1 ml of 0.1 M citrate buffer pH 5.5. Then 0.5 mg of 11α-hydroxyprogesterone-hemisuccinate in 1 ml solution was added with 0.1 ml of 1 M carbodiimide, the pH of which had been previously adjusted to 4.7. After 30 min of incubation at room temperature, 5 mg of β-galactosidase in 1 ml of 0.1 M citrate buffer pH 5.5 was added to the mixture. The pH was brought to 5.5, and the solution was incubated at 4°C for 12 h.

The mixture was added with 10 ml of 0.1 M phosphate buffer pH 7.0 to stop the reaction and was dialysed against 2 l of 0.1 M phosphate buffer at 4°C overnight.
The final volume of the conjugate was 12.1 ml. The conjugate solution was concentrated to 5.5 ml using Diaflow PM 30 membrane (Amicon).

One ml of the conjugate was chromatographed on a Sephadex G-25 column (10 mm x 145 mm). The column was eluted with 20 ml of 0.1 M phosphate buffer pH 7.0. Each fraction of 1 ml of the eluate was collected, and a small portion of each fraction was diluted 2000-fold with 0.1 M phosphate buffer pH 7.0, and the enzyme activity was measured. The enzyme activity of each fraction indicates an elution pattern of the conjugate as a single peak.

The fraction with the highest activity was diluted 2-fold with 0.1 M phosphate buffer pH 7.0 and stored at 4°C. This conjugate was stable at least 16 months as regards both enzyme activity and immunoreactivity. For enzyme immunoassay, the solution diluted 200-fold with the same buffer was used.

7. Procedure for enzyme immunoassay.

Serum (0.1 ml–0.5 ml) was extracted with 20 volumes of petroleum ether by shaking for 30 s on a Yamato touch mixer (Yamato Scientific Co., Ltd., Tokyo). The ether layer was transferred to another tube and dried. 0.1 ml of 0.1 M phosphate buffer pH 7.0 was added and mixed in the mixer for 5 s. This was added with 0.1 ml of progesterone antiserum (× 8000). After gentle shaking, the mixture was incubated at room temperature for 3 h. Then 0.1 ml of progesterone-β-galactosidase conjugate solution was added and allowed to react at room temperature for 3 h. At the end of the incubation 0.1 ml of anti-rabbit gamma globulin goat serum (second antibody) (× 40) was added. The solution was then incubated at 4°C overnight. After centrifugation at 3500 r.p.m. for 20 min, the precipitate was washed once with 2 ml of 0.1 M phosphate buffer and resuspended in 2 ml of the same buffer. Enzyme activity was measured as described earlier.

Progesterone dissolved in methanol was used as standard. 0, 25, 50, 100, 250, 500 and 1000 pg in 0.1 ml or 0.2 ml of methanol were dried and added together with 0.1 ml of 0.1 M phosphate buffer pH 7.0 and thereafter treated in the same manner as serum samples.

A standard curve was obtained by plotting absorbance against the amount of progesterone added.

8. Procedure for radioimmunoassay

Procedure for radioimmunoassay was essentially the same as the method of Makino (1973).

Results

1. Antiserum dilution

Antiserum to progesterone was diluted serially from ×4000 to ×64 000 except ×8000. Standard curves obtained using the serially diluted antisera are shown in Fig. 1. In this experiment, second antiserum was used without dilution, while the second antibody was usually used at the dilution of 1:40. It is indicated that anti-progesterone serum may be used at a dilution between ×4000 and 16 000. Anti-progesterone serum at a dilution of 1:8000 was generally used for the enzyme immunoassay. When antiserum to progesterone-3-oximbovine serum albumin instead of the above mentioned antiserum to 11α-hydroxyprogesterone-hemisuccinate-bovine serum albumin in the assay was used, no inhibition of binding of progesterone-β-galactosidase conjugate and progesterone antiserum by free progesterone was observed.

2. Standard curve

An example of standard curves for progesterone enzyme immunoassay is shown in Fig. 2. The least detectable concentration of progesterone (concentration resulting in a response two standard deviations away from the zero dose response) was estimated as 12 pg. Blank values determined by using 0.1 ml of distilled water instead of serum were usually below 20 pg. The sensitivity of this immunoassay was considered to be 25 pg/tube.
Fig. 2.
Standard curve for enzyme immunoassay of progesterone. Note: each point represents mean ± sd from 5 replicate assays.

3. Recovery of progesterone added to serum
The recovery rate of 0.05, 0.10, 0.25, 0.50, and 1.00 ng of progesterone added to 0.10, 0.25, and 0.50 ml of pooled bovine serum averaged 98.1±11.4% (mean ± sd) as shown in Fig. 3.

Table 1.
The cross reaction of antiserum to 11α-hydroxyprogesterone hemisuccinate-bovine serum albumin with various steroids determined by enzyme immunoassay and radioimmunoassay.

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Cross reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>21.63</td>
</tr>
<tr>
<td>5α-pregnane-dione</td>
<td>12.77</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>10.39</td>
</tr>
<tr>
<td>20α-hydroxyprogesterone</td>
<td>3.12</td>
</tr>
<tr>
<td>17α-hydroxyprogesterone</td>
<td>2.00</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.56</td>
</tr>
<tr>
<td>Others</td>
<td>&lt; 0.53</td>
</tr>
</tbody>
</table>

4. Cross reaction
The cross reaction of the progesterone antiserum with other steroids determined by the enzyme immunoassay and radioimmunoassay are shown in Table 1.

5. Precision analysis
Precision of the enzyme immunoassay was analyzed on the pooled serum samples obtained from 2 cows in the follicular and luteal phases.

Fig. 3.
The recovery of various amounts of progesterone added to bovine serum by enzyme immunoassay.

Fig. 4.
Correlation between progesterone concentrations in bovine serum measured by enzyme immunoassay and radioimmunoassay.
The intra-assay coefficient of variation in the serum obtained in the follicular phase was 6.7% (n = 5, mean = 0.46 ng/ml) and it was 9.4% (n = 5, mean = 3.24 ng/ml) in the serum collected in the luteal phase, respectively. The inter-assay coefficient of variation was 8.7% (n = 5, mean = 0.45 ng/ml) and 4.7% (n = 5, mean = 3.48 ng/ml), respectively.

6. Correlation between progesterone concentrations in the serum measured by enzyme immunoassay and radioimmunoassay.

Progesterone levels in the serum of 17 cows with cystic ovaries estimated by enzyme immunoassay were highly correlated (r = 0.99, P = 0.01) with the values obtained by radioimmunoassay (Fig. 4).

Discussion

Concentration of progesterone in the peripheral blood serum is the most useful indicator of luteal function. This study described a specific enzyme immunoassay of progesterone which was highly sensitive and simple. The assay system requires only a centrifuge and spectrophotometer. This enables the most of clinical laboratories to undertake an immunoassay of progesterone.

The minimum concentration of progesterone which can be determined by this method is 12 pg, a little lower than reported by Dray et al. (1975) and significantly lower than that by Joyce et al. (1977). Since the blank value was below 20 pg, the measurable range of progesterone in 0.1 ml of serum was considered to be between 0.25 ng/ml and 10 ng/ml. If 0.5 ml of serum was used, the sensitivity would be 0.05 ng/ml.

This sensitivity is sure to satisfy the requirements for a practical assay method of progesterone in the serum of various species of animals and in man.

All the data concerning the recovery of progesterone added to serum, the cross reactivity of the antiserum with other steroids, and the precision in this assay further indicate that the enzyme immunoassay is applicable as a practical method of progesterone measurements.

Acknowledgments

The author wishes to thank Dr. A. Yuasa of Physiological Department of this university and Dr. A. Kanbegawa of Teikoku Hormone Mfg. Co. Ltd., Tokyo, for their most valuable advice and criticism.

The thanks are also due to Dr. K. Kawata, professor of the author's department for his interest and encouragement in this study.

References


Received on April 9th, 1979.